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DEVELOPMENT OF NOVEL EST-SSR MARKERS TO ASSESS GENETIC DIVERSITY IN CURCUMA LONGA L

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ABSTRACT

Curcumalonga (turmeric) belonging to the family Zingiberaceae has been widely used as medicinal plants, spices and ascosmetics. For crop improvement and conservation, understanding the genetic diversity among turmeric accessions is an important goal. Therefore, in this study, the Expressed Sequence Tag-Simple Sequence Repeat marker (EST-SSR) was used to identify due to distinguish between heterozygous and homozygous loci and genetic diversity analysis 24 accessions of Curcuma longa, 2 accessions of C. manga and 2 accessions of C. zedoaria. From 23 EST-SSR primers were designed from EST database and tested. The result showed that 4 primers (EST7, EST10, EST14 and EST18) which provided a good amplification and polymorphic bands. A total of 33 alleles were detected among all the C. longa accessions. The similarity coefficient using NTSYS pc (version 2.20e) ranged from 0.25–1.00. A dendrogram was also constructed by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) which divided C. longa into four clusters according to geographical origin. Therefore, the development of EST-SSR markers is useful for determining the genetic diversity of C. longa.

KEYWORDS: Curcuma Longa, Microsatellite Marker & Genetic Diversity

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INTRODUCTION

***Curcuma longa* L.** (Turmeric) is the best-known species in family Zingiberaceae which consists of more than 80 species and distributed in Asia, South East Asia, and Africa. (Joshi et al., 2010). According to its several benefits as a food flavoring agent, food coloring agent, food preservation, medicinal uses, and cosmetics, several types of substances and essential oil are investigated. Moreover, Curcumin was an important chemical which is capable of antioxidant and others reported as anti-inflammatory, antibacterial, gastrointestinal and respiratory disorders (Cousin et al., 2006). Hence, Turmeric has been long time worldwide valuable herb and Thailand is one of the producing and exporting leaders of Turmeric. Expansive cultivation and unsuitable crop improvement lead to sterile and indistinguishable traits. Various studies on the morphological and biochemical character have been introduced earlier but there is a lack of information toward phylogenetic relationship among *Curcuma* in Thailand (Taheri et al., 2012). The relationship of *Curcuma* using morphology to identify in different verities is slow and vague compared to molecular markers which have been useful and accelerated breeding programs.

Microsatellites or simple sequence repeats (SSRs) are a tandem repeat of short 1–6 base pairs DNA. Microsatellites is co-dominant marker which is uncomplicated because the result can be rapidly analyzed by polymerase chain reaction (PCR) and gel electrophoresis. In addition, SSR loci are distributed over the genome and provide high information (Joshi et al., 2010). The former method to develop genomic SSR is high time consuming and labor intensive. Recently, Microsatellites could be derived from expressed sequence tags (ESTs)

database which is the short sequence from mRNA (cDNA). EST-based SSR had been used asa molecular marker for genetic diversity, cultivar identification and fingerprinting. Several studies have been conducted EST-SSR as a molecular marker in plants such as *Ocimum basilicum, Apium graveolens L. and Porphyra* (Gupta et al., 2010, Fu et al., 2013, Sun et al., 2006). The results gave good amplification patterns, separated the cultivars into clusters which related to originated geographicaldata. Moreover, the genetic diversity based on EST-SSR has also done in endemic *Curcuma* in Chhattisgarh, India and it can be efficiently amplified for identification in Curcuma species (Jain and Parihar, 2019). In Thailand, *C. longa* EST-SSR information is limited. Thus, in this study, EST-SSR was developed to enhance genomic studies and identify genetic relationship in genus *C. longa* in Thailand.

MATERIALS AND METHODS

Plant Material and DNA Extraction

The Stem of 24 accessions of *C. longa*, 2 accessions of *C. manga* and 2 accessions of *C. zedoaria* were collected from Northern Southern and Northeast of Thailand, as shown in table 1. Total genomic DNA was extracted by using the modified CTAB method **(**Doyle and Doyle, 1987) and DNA were detected on 0.8% agarose gel and spectrophotometer.

EST-SSR Primers Design

12,768 the EST sequences of *Curcuma longa* were retrieved from dbEST database of NCBI (National Centre for Biotechnology Information, https://www.ncbi.nlm.nih.gov/). The presence of EST-SSR repeat with a minimum repeat number of three and mono-, di-, tri-, tetra-, penta- and hexa-nucleotides were screened with the online software MISA (http://pgrc.ipk-gatersleben.de/misa/misa.html). EST-SSR primers sequences were designed using the Primer 3 plus software program. A total of EST-SSR primers were synthesized (Macrogen, Korea) and used in this study.

PCR Amplification and Data Analysis

EST-SSR amplification was conducted in 25 ml of reaction mixture containing 10X PCR buffer, 2.5 mM $MgCl_2$, 0.1 mM dNTP, 200 nM primers, 50 ng template DNA, and 1.0 U *Taq* DNA polymerase (Thermo Scientific). Amplifications were made in a Perkin Elmer 9600 thermocycler with an initial denaturing step of 3 min at 94 °C, followed by 35 cycles of 30 s at 94°C, 45 s at 52°C-55 °C, 45 s at 72°C and a final extension of 5 min at 72°C. PCR products were separated by electrophoresis on 3% agarose gels in TBE buffer and visualized using ethidium bromide staining. The presence or absence of every single fragment was coded as 1 or 0, respectively, and scored for a binary data matrix. Similarity was estimated byNei and Li (1979) and a dendrogram based on similarity coefficients was generated using the unweighted Pair Group Method with Arithmetic Mean (UPGMA) in NTSYSpc 2.2 (Rohlf, 2000). The polymorphism information content (PIC) was calculated according to equation $PIC = 1-\Sigma pi^2$, where Pi is the frequency of the i-th allele. PIC values range from 0-1 (Smith *et al*., 1997).

Table1: List of 28 Curcuma Accessions Included in this Study

Accession number	Species	Location
CU1	Curcuma longa	Chiang Mai
CU2	Curcuma longa	Chiang Mai
CU3	Curcuma longa	Phitsanulok
CU4	Curcuma longa	Phitsanulok
CU5	Curcuma longa	Lampang
CU6	Curcuma longa	Lampang
CU7	Curcuma longa	Lampang

Table 1: Contd.,		
CU8	Curcuma longa	Lamphun
CU9	Curcuma longa	Lamphun
CU10	Curcuma longa	Lamphun
CU11	Curcuma longa	Ranong
CU12	Curcuma longa	Ranong
CU13	Curcuma longa	Phitsanulok
CU14	Curcuma longa	Nakhon Ratchasima
CU15	Curcuma longa	Uttaradit
CU16	Curcuma longa	Phrae
CU17	Curcuma longa	Phayao
CU18	Curcuma longa	Phayao
CU19	Curcuma longa	Chiang Rai
CU20	Curcuma longa	Chiang Rai
CU21	Curcuma longa	Chiang Rai
CU22	Curcuma longa	Chiang Rai
CU23	Curcuma longa	Chiang Rai
CU24	Curcuma longa	Chiang Rai
CU25	Curcuma zedoaria	Lamphun
CU26	Curcuma zedoaria	Chiang Mai
CU27	Curcuma mangga	Lampang
CU28	Curcuma mangga	Lamphun

RESULTS AND DISCUSSIONS

A total of 35 EST-SSR primers were designed from EST sequences of *Curcuma longa*, of these, 23 pairs of primers gave clear bands (table 2). Most primers contained only one SSR, a few represented two, four or five SSRs. From 23 SSR containing sequences, tri-nucleotides were the most common at 52% agreement with the previous study that showed trinucleotide SSRs being the most abundant type in *C. longa* ESTs (Kumar *et al*., 2010). However, only 4 primers (EST7, EST10, EST14 and EST18) showed polymorphism that detected 33 alleles and the number of alleles per locus varied from 7 to 12. These primers produced multiple banding patterns due to *C. longa* had polyploidy chromosomes in accordance with previous reports (Sahoo *et* al., 2017; Taheri *et al*., 2019). The Product size of EST18 primer represented larger than the expected size that might be resulted from the amplification of intron (Sahoo *et* al., 2017). The 23 EST-SSR primer pairs developed from ESTs of *Curcuma longa* were successfully amplified in *C. zedoaria* and *C. mangga* that were used as out-group. PIC values were presented in table 3. Four primers represented a high polymorphic information content (PIC) value, on average 0.84, suggesting sufficient discriminatory power for determining the genetic diversity and relationships among *C. longa*. In contract on, average PIC score using 17 EST-SSRs from *C. longa*, *C. cassia* and *C. aromatica* was 0.38 (Jain and Parihar, 2019). In addition, 4 ESTs had homology with known proteins that were performed by BLASTX (table 3).

The EST-SSR primers were used in genetic diversity analysis of *C. longa* accessions. The dendrogram was constructed through UPGMA algorithm using Jaccard's similarity coefficient through NTSYSpc 2.2 software (figure. 1). The coefficient of similarity index showed most of the accessions shared 0.25–1.00, indicating that the accessions were highly different in their genetic characteristics. According to Verma *et al*. (2015) studied the genetic variability in indigenous turmeric germplasm using DAMD and ISSR that this genotype also revealed high genetic diversity. All 24 accessions were group into four clusters. The dendrogram clearly separated *C. longa* from out-group and the most clusters represented distinct clusters by region. The highest genetic similarity value (1.00) was observed between CU6 and CU7 as same as CU11 and CU12 that they were collected from the same province. Cluster I contained 5 samples from the northern

part (Chaing mai and Lumpang). Cluster II consisted of ten turmeric samples that were placed in several provinces. In addition, CU17, CU19, CU20, CU21 CU22, CU23 and CU24 were group into cluster III with the accession from Phayao and Chiang Rai. Cluster IV consisted of 2 accessions (CU6 and CU7) collection from Lampang that showed the high genetic similarity (1.00).

Table 2: Primer Sequences, Repeat Motif and Expected Size for Newly Developed EST-SSR

Primer	Primer Sequence (5'–3′)	Repeat Motif	Expected Size
EST1	F: GCTGGTTGCAGATATCAGTA	$(AAAC)_6$	201
	R: CCAAAGGTTCCCCTCCAT		
EST2	F: ATGAGGGAAGAGAGGAGAAG	$(AG)_{15}$	217
	R: GCAACTTTCTCACTGGTTCC		
EST3	F: GCATACATTGACGAAGAGGAA	$(ATT)_7$	236
	R: GATAAAGCCATAATACCATTCCTC		
EST4	F:CAGAGGGCATTTCCACAAGT	$(TA)_7$	224
	R: AACACGGGCGCATAAAAATTTG		
EST5	F: GTCTTTATGCCACGGGCGC	$(TA)_7$	222
	R: CCACAAGTGCGAGCTGAGAAA		
EST6	F: CTTGGTAGACGTTCCCGCAA	$(CG)_8$	222
	R: GGACGGAAGTCGTAATCTGG		
EST7	F: GTCGCTAAACATGGATGCAT	$(AAG)_{12}$	206
	R: TGATTAAGGACAAGGGAGCA		
EST8	F: CAAGCTCCAAATAAGTCAAGAT	$(AAT)_7$	204
	R: TGCATACTATCGCAAAAGGTG		
EST9	F: TACAACCCTCCTCCCATTAG	$(AGG)_7$	205
	R: CCGCCTTCCGACACTTTATA		
EST10	F: ATCAGAAACTTTCAGAATAACCAG	(ATCATC)4	252
	R: GGAACAAAAGGAAGACTATTCAT		
EST11	F: GACAATCCAAAGCATGCGCT	(GCT)7	245
	R: CAGAGCAAGGGCCATGGT		
EST12	F: GCCCCCCCCAAAAATTTTCC	$(A)_{12}$	210
	R: TGGTTTCCCGCCTTTTTTAGAG		
EST13	F: GAGAAGAACAAGGCTTGTCG	(AG)7	120
	R: CGTCTTGGAGCTTGCCTC		
EST14	F: AAGCAGTTGATCAATGGCCG	(CCT)6	218
	R: GCGAGGCGGCAAGGAG		
EST15	F: CTCTGCTCCTTCAACCAGTT	(AAT)8	210
	R: GCTGCCTAAACATCTACCTC		
EST16	F: GCAGATGCTATGGTTACATG	(ATT)8	214
	R: TCAAGTTTGAAGGATTACAGTCA		
EST17	F: TAC ATGGAGTGATAAGTAATTACG	(TAGA)8	218
	R: CTCTCCGTTTGTAATTCGTCG		
EST18	F: CAGCGTTGTTACATGGCACC	(AAAC)6	210
	R: TTCCCCTCCATGAAACTCCA		
EST19	F: GAAGAGAGGAGGTTTGGGGA	(GCG)3, (CGA)3	278
	R: TGATATTCCATAAGCTCAAACAGG		
EST20	F: ATCAATAGTTACGATGACAATCGA	(CTTGCC)3, (TC)3	265
	R: ACTCGGTTGACGTCTGGTC		
EST21	F: GCGAAGAGCAGCTGCCT	(C)6, (CCT)3, (GCA)5, (TGA)3	362
	R: TTGAGCACCCTCCTCTTGA		
EST22	F: CTCATGGAGGAGCTCTGCA	(GAC)3, (GGCGAC)6, (C)6,(A)8, (T)8	368
	R: ACTGGCGAGAGCTCAATTTCA		
EST23	F: AACAATCGTTCGCCG GGATA	(CGC)3, (AT)4, (AAT)3, (TA)4	315
	R: TATAGGCCGTTAAGTACTCGAT		

Table 3: Characteristics of Primers for Analyzing Genetic Diversity of C. Longa

Primer	PIC	Size Range (Bp)	Expected Size	Allele	Putative Function; Accession Code
EST7	0.88	220-300	206	12	UDP-glucose 6-dehydrogenase 5-like (LOC103987519)
EST10	0.97	290-350	252	7	photosystem I reaction center subunit III, chloroplastic-like (LOC103989585)
EST14	0.77	200-300	218	7	ethylene-responsive transcription factor ERF027-like (LOC103716877)
EST18	0.74	400-450	210	7	E3 ubiquitin-protein ligase XBOS32 (LOC103980290)
Mean	0.84	-	-	8.25	-

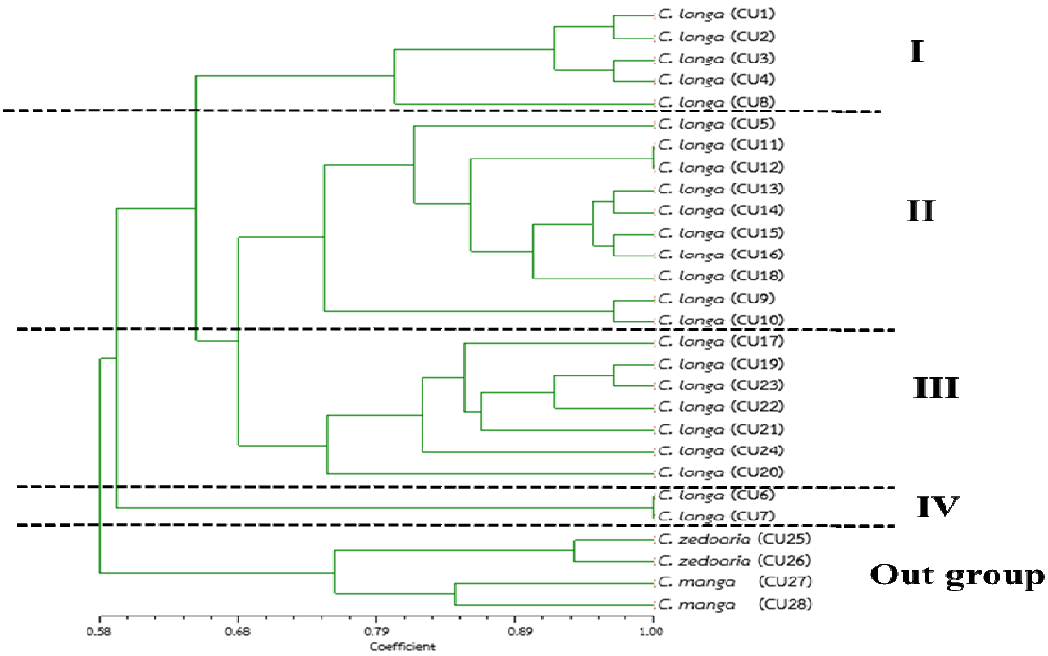

Figure 1: Dendrogram Based on UPGMA, Representing The Genetic Relationship among the C. Longa Accessions using EST-SSR Markers.

CONCLUSIONS

The EST-SSR markers developed for 26 accessions of *C. longa* and these markers were also used in *C. zedoaria* and *C. mangga*. This research focused on developing EST-SSR markers for genetic diversity study. However, there were only 4 primers i.e., EST7, EST10, EST14 and EST18 which generated polymorphic alleles. The high average PIC values indicated that the primers were able to discriminate among *C. longa* genotypes. In addition, the number of alleles for polymorphic markers ranged from seven to twelve with a mean of 8.52. The dendrogram was constructed by NTSYSpc-2.20 k, UPGMA method and the similarity index ranged from 0.25–1.00 which separated 26 accessions of *C. longa* into 4 groups.

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